British Journal of Pharmacology (2001) 134, 529–534

www.nature.com/bjp

Orphanin FQ/nociceptin attenuates the development of morphine tolerance in rats

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1 Recent evidence from studies in mice lacking the opioid receptor-like (ORL-1) receptor and from experiments using antibodies raised against orphanin FQ/nociceptin (OFQ/N) suggest that this peptide may be involved in morphine tolerance. In the present study we sought to investigate if administration of exogenous OFQ/N would modulate the development of tolerance to the antinociceptive effect of morphine.

2 Rats were treated for 3 days with either saline or morphine (10 mg kg^{-1}, s.c.) followed, 15 and 75 min later, by two intracerebroventricular injections of either artificial cerebrospinal fluid (aCSF) or OFQ/N. The dose of OFQ/N was doubled each day (7.5, 15, 30 nmol). On day 4, rats were tested on a hot plate apparatus before and 30, 60 and 90 min after morphine administration.

3 Repeated OFQ/N treatment did not affect basal nociceptive responses or morphine-induced antinociception. However, the same treatment significantly attenuated the development of morphine tolerance.

4 Since learning and memory could contribute to the development of morphine tolerance, in subsequent studies, we examined the effect of OFQ/N administered in the CA3 region of the hippocampus, where OFQ/N has been shown to block LTP and impair spatial memory. A greater attenuation of morphine tolerance with no alteration of baseline hot plate latency or morphine-induced antinociception was observed when OFQ/N was administered in this area of the rat brain.

5 Taken together, our results demonstrate that OFQ/N may act in the hippocampus to attenuate morphine tolerance.

British Journal of Pharmacology (2001) **134,** 529–534

Keywords: Morphine; orphanin FQ/nociceptin; antinociception; tolerance; CA3 region; hippocampus; rat hot plate test

Abbreviations: aCSF, artificial cerebrospinal fluid; cyclic AMP, adenosine-3′,5′-monophosphate; i.c.v., intracerebroventricular(ly); LTP, long-term potentiation; OFQ/N, orphanin FQ/nociceptin; ORL-1, opioid receptor-like

Introduction

Opioid analgesics, such as morphine, are widely used for treatment of moderate to severe pain. In addition to side effects such as respiratory depression and constipation, the clinical usefulness of these drugs is often hampered by the development of tolerance with chronic administration (for review, see Osenbach & Harvey, 2001). The mechanism of morphine tolerance, defined as a decrease in the potency of the drug with repeated use, is not fully understood. At the receptor level, possible mechanisms of tolerance may involve opioid receptor desensitization (Law *et al*., 1982) and/or down-regulation (Law *et al*., 1982; Puttfarcken *et al*., 1988). In addition to receptor regulations, a change in the level of second messenger systems has been reported in opioid-tolerant subjects. Chronic opioid treatment, for instance, leads to an increase in intracellular calcium levels (Welch & Bass, 1995; Smith *et al*., 1999). Moreover, elevated activity of certain enzymes, such as adenylyl cyclase (Sharma *et al*., 1975; Klee *et al*., 1984), nitric oxide synthase (Kolesnikov *et al*., 1993), adenosine-3′,5′-monophosphate (cyclic AMP)-dependent protein kinase (Wang *et al*., 1996; Bernstein & Welch, 1998; Wang & Sadee, 2000) and protein kinase C (Mao *et al*., 1995; Mayer *et al*., 1995; Narita *et al*., 1995) have been associated with opioid tolerance. However, such mechanisms alone are not sufficient to explain some forms of tolerance. For instance, morphine produces tolerance but does not induce down-regulation (Tao *et al*., 1987; Yoburn *et al*., 1993; Keith *et al*., 1996) or internalization of opioid receptors (Arden *et al*., 1995; Keith *et al*., 1996).

Among other proposed mechanisms is the increased activity of so-called anti-opiate peptides in the brain (for reviews, see Harrison *et al*., 1998; Goodman *et al*., 1998). One recently discovered peptide with counter-opiate action is orphanin FQ (OFQ; also known as nociceptin), an endogenous ligand of the opioid receptor-like (ORL-1) receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995). Similarities in the structures of the ORL-1 receptor and the heptadecapeptide OFQ/N with those of opioid receptors and endogenous opioid peptides, respectively, indicate a close evolutionary relationship between the two systems (Mollereau *et al*., 1994; Meunier *et al*., 1995; Reinscheid *et al*., 1995). Despite the resemblance, OFQ/N does not bind to the classical opioid receptors (Mollereau *et al*., 1994). More importantly, although OFQ/N produces the same cellular effects as classical opioids, such as inhibition of cyclic AMP (Meunier *et al*., 1995; Reinscheid *et al*., 1995), inhibition of

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calcium channel conductance (Knoflach *et al*., 1996; Connor *et al*., 1996b), and an increase in potassium efflux (Matthes *et al*., 1996; Vaughan & Christie, 1996; Connor *et al*., 1996a), intracerebroventricular injection of OFQ/N produces hyperalgesia (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Calo *et al*., 1998; Lutfy & Maidment, 2000) and attenuates the antinociceptive effects of opioid analgesics (Mogil *et al*., 1996; Tian *et al*., 1997; Lutfy *et al*., 1999; Wang *et al*., 1999).

Regarding the role of OFQ/N in opioid tolerance, a recent study showed increased tissue and ventricular cerebrospinal fluid levels of OFQ/N in the brains of morphine tolerant rats (Yuan *et al*., 1999). On this basis it has been proposed that continual administration of morphine accelerates the biosynthesis and/or release of OFQ/N to antagonize the effect of morphine, thereby contributing to the phenomenon of tolerance. In support of this idea, intracerebroventricular (i.c.v.) injection of an antibody raised against OFQ/N partially reversed the expression of morphine tolerance (Tian *et al*., 1998). Moreover, morphine tolerance is partially inhibited in mice lacking the nociceptin receptor gene (Ueda *et al*., 1997), raising the possibility that the ORL-1 receptor may play a functional role in the development of morphine tolerance. The present study was designed to evaluate the effect of exogenously administered OFQ/N on the development of morphine tolerance. The first experiment employed an i.c.v. route of administration and provided evidence for an OFQ/N-induced attenuation of the development of morphine tolerance. Given that morphine tolerance and learning and memory may share common mechanisms (Siegel, 1976), and since OFQ/N attenuates long-term potentiation (LTP) in the hippocampus (Yu *et al*., 1997) and also impairs spatial memory in a water-maze test when injected directly into the CA3 region of the rat hippocampus (Sandin *et al*., 1997), the second experiment employed local injection of OFQ/N in this brain region and similarly showed blockade of morphine tolerance.

Methods

Subjects

Male Sprague Dawley rats (180–200 g) obtained from Harlan (San Diego, CA, U.S.A.) were maintained two per cage, with free access to food and water. All experiments were conducted during the light phase of a 12-h/12-h light/dark cycle.

Surgery

Rats were anaesthetized with halothane in a 1 : 1 mixture of oxygen and nitrous oxide, and implanted with a 22-G guide cannula (3.5-mm long) 0.5 mm above the right lateral ventricle or the CA3 region of the hippocampus. The coordinates for intracerebroventricular and intra-hippocampal injection sites were, according to the atlas of Paxinos and Watson (1986): AP = −0.8 mm, ML = +1.4 mm; DV = −4.0 mm; AP = −3.3 mm; ML = ±2.4 mm, DV = −4.0, respectively. A 30-G needle was used for artificial cerebrospinal fluid (aCSF) or OFQ/N injection.

Drug treatment schedule

Rats were allowed 4 days to recover from the surgical procedure and then treated once daily for 3 days. On day 1, rats received a subcutaneous (s.c.) injection of either saline (SAL) or morphine (MOR; 10 mg kg^{-1}). After 15 min, rats were given an injection of either aCSF or OFQ/N either in the right lateral ventricle (7.5 nmol in 5 μl) or directly into the CA3 region of the hippocampus (7.5 nmol in 0.5 μl per side). Based on previous experience with this peptide, which indicated a short duration of action in the brain, we administered an additional dose of aCSF or OFQ/N 1 h later. On days 2 and 3, the same respective treatments were given, except that the dose of OFQ/N was doubled each day (15 nmol on day 2 and 30 nmol on day 3) because tolerance develops to the anti-opioid action of exogenous OFQ/N (Lutfy *et al*., 1999). On day 4, rats were tested for baseline nociceptive responses in the hot plate test (for details, see below). Approximately 30 min later, all groups received an injection of morphine (10 mg kg^{-1}, s.c.) and were tested for post-drug hot plate latency at 30-min intervals for 90 min.

Nociceptive assay

A modification of the hot plate assay of Woolfe & MacDonald (1944) was used. Rats were placed on a warm plate (52°C) surrounded by a Plexiglas cylinder and the amount of time taken for the rats to lick/flutter one of the hind-paws or jump from the plate was measured. A cut-off time of 60 s was employed in order to prevent tissue damage.

Verification of guide cannula placement

At the end of each experiment, rats were anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.) and perfused transcardially with phosphate buffered saline (PBS) containing NaCl (130 mM); NaH2PO4 (3 mM) and Na2HPO4 (7 mM) followed by 50 ml of phosphate buffered formalin. Brains were removed and sectioned (40 μm) using a cryostat (Leica Instruments Gmbh, Germany). The slices were stained using cresyl violet and viewed under the microscope. Based on such examination, five rats were discarded from the data analysis due to improper placement of the guide cannula.

Data analysis

Hot plate latencies were analysed using a two-way analysis of variance (ANOVA) with repeated measures on the hot plate latency at different time points. The Newman-Keuls *post-hoc* test was used to examine the significance of differences among various groups. A value of $P<0.05$ was considered statistically significant.

Drugs

Morphine sulphate was obtained from Mallinckrodt, Inc. (St. Louis, MO, U.S.A.) and dissolved in normal saline prior to s.c. injection. OFQ/N was purchased from Phoenix Pharmaceuticals, Inc. (Mountain View, CA, U.S.A.) and dissolved in aCSF prior to i.c.v. application. The composition of aCSF in mM was: NaCl 125, KCl 2.5, NaH2PO4

0.9, Na2HPO4 5, MgCl2 1, D-glucose 2.5, CaCl2 1.2, bovine serum albumin 0.025%.

Results

Effects of i.c.v. OFQ/N treatment on the development of morphine tolerance

Morphine administration on day 4 produced a significant ($F_{3,105} = 87.87$; $P < 0.05$) antinociceptive effect (Figure 1), the magnitude of this response was dependent on prior treatment on days 1–3 ($F_{3,35} = 15.94$; $P < 0.05$). The *post-hoc* Newman-Keuls test revealed that treatment with morphine (10 mg kg^{-1}, s.c.) for 3 days induced tolerance to its antinociceptive effect when rats were tested on day 4 ($P < 0.05$; SAL–aCSF *vs* MOR–aCSF). This tolerance to morphine was significantly attenuated in rats treated for 3 days with morphine followed by OFQ/N ($P < 0.05$; MOR–OFQ *vs* MOR–aCSF). There was no significant difference in baseline values among the four groups ($P > 0.05$), indicating that chronic treatment with morphine or OFQ/N, either alone or in combination, did not affect the basal nociceptive response in the hot plate test. Moreover, chronic treatment with OFQ/N alone did not change the antinociceptive effect of morphine in the hot plate test ($P > 0.05$; SAL–aCSF *vs* SAL–OFQ).

Effects of intra-hippocampal OFQ/N treatment on the development of morphine tolerance

A similar pattern of effects was observed with intra-hippocampal OFQ/N administration (Figure 2). Again, morphine produced a significant antinociceptive effect on day 4 ($F_{3,57} = 79.55$; $P < 0.05$), the magnitude of which was dependent on prior treatment on days 1–3 ($F_{3,19} = 16.91$; $P < 0.05$). *Post-hoc* analysis of the data showed that morphine treatment for 3 days induced tolerance to the antinociceptive effect of the drug on day 4 ($P < 0.05$; SAL–aCSF *vs* MOR–aCSF) and the extent of such tolerance was attenuated by concomitant intra-hippocampal treatment with OFQ/N ($P < 0.05$; MOR–OFQ *vs* MOR–aCSF). Indeed, tolerance was effectively blocked by intra-hippocampal OFQ/N treatment since there was no significant difference between rats treated with morphine plus OFQ/N and saline-treated control groups ($P > 0.05$; MOR–OFQ *vs* SAL–OFQ or *vs* SAL–aCSF).

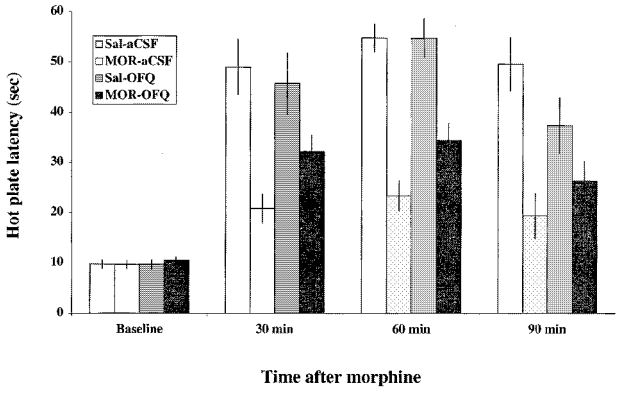

Figure 1 Effects of intracerebroventricular (i.c.v.) OFQ/N administration on morphine tolerance in the rat hot plate test. Rats were treated for 3 days with saline or morphine (10 mg kg^{-1}, s.c.) followed, 15 and 75 min later, by two i.c.v. injections of aCSF or OFQ/N. On day 4, rats were tested 30 min before and 30, 60 and 90 min after morphine (10 mg kg^{-1}, s.c.) in the hot plate test. Treatment with morphine for 3 days produced a significant decrease in the antinociceptive effect of the drug ($P < 0.05$, SAL–aCSF vs MOR–aCSF) and this tolerance was attenuated by i.c.v. OFQ/N ($P < 0.05$, MOR–OFQ vs MOR–aCSF) administration (*post hoc* Newman-Keuls test). Data were analysed by a two-way ANOVA with repeated measures on hot plate latencies before and after morphine administration on day 4.

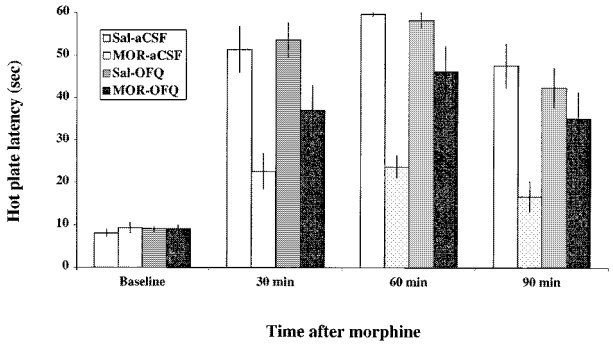

Figure 2 Effects of intra-hippocampal OFQ/N treatment on morphine tolerance in rats using the hot plate test. Rats received the same treatment and were tested as described in the legend to Figure 1 except aCSF or OFQ/N was administered into the CA3 region of the hippocampus. Morphine treatment for 3 days induced tolerance ($P < 0.05$, SAL–aCSF vs MOR–aCSF) and this phenomenon was blocked by administration of OFQ/N ($P < 0.05$, MOR–OFQ vs MOR–aCSF) into the CA3 region of the rat hippocampus (*post hoc* Newman-Keuls test). Data were analysed by a two-way ANOVA with repeated measures on hot plate latencies before and after morphine administration on day 4.

Discussion

The major finding of the present study is that chronic intracerebroventricular or intra-hippocampal treatment with OFQ/N attenuated the development of morphine tolerance without affecting the basal nociceptive response or acute antinociceptive effect of the opioid analgesic in the rat hot plate test. Taken together, our results indicate that repeated OFQ/N administration selectively interferes with the processes involved in the development of morphine tolerance.

Numerous studies have reliably shown that tolerance develops to the antinociceptive effect of morphine after chronic use of the drug (see, Cox, 1990). The mechanism of this phenomenon is yet to be fully characterized, but one proposal, among many, is an increase in the activity of anti-opioid peptides in the brain (for reviews, see Goodman *et al.*, 1998; Harrison *et al.*, 1998). For instance, chronic morphine treatment has been associated with an increase in immunoreactivity for cholecystokinin (Lucas *et al.*, 1999; Becker *et al.*, 2000) and neuropeptide FF (Malin *et al.*, 1990), both of which have been shown to oppose actions of opioid analgesics in the central nervous system. The demonstration that OFQ/N blocks opioid receptor-mediated stress-induced antinociception (Mogil *et al.*, 1996) and also decreases the antinociceptive effect of morphine and other opioid receptor

agonists (Mogil *et al*., 1996; Morgan *et al*., 1997; Tian *et al*., 1997; Lutfy *et al*., 1999; Wang *et al*., 1999) has led to the suggestion that OFQ/N may be acting as an anti-opioid peptide in the brain (Mogil *et al*., 1996).

The involvement of OFQ/N in morphine tolerance was recently supported by the observation that the tissue concentration of endogenous OFQ/N increased in the rat periaqueductal gray and amygdala in a time-dependent manner during the development of morphine tolerance (Yuan *et al*., 1999). The OFQ/N concentration was also elevated in the ventricular cerebrospinal fluid of these animals (Yuan *et al*., 1999). Such a combination of results indicates a gradual increase in OFQ/N biosynthesis and/or release during the development of morphine tolerance. The result of a previous study (Tian *et al*., 1998) showing that the expression of morphine tolerance was attenuated by acute administration of an antibody raised against OFQ/N is consistent with a causal relationship between elevated OFQ/N release and morphine tolerance. Such a conclusion is further strengthened by the observation that ORL-1 knockout mice display only partial tolerance to morphine compared to wild-type mice (Ueda *et al*., 1997).

Our data show that exogenous OFQ/N, administered concomitantly with repeated morphine injections, attenuated the development of morphine tolerance. On the surface, this may seem to conflict with the data of Tian *et al*. (1998) using an OFQ/N antibody. However, it should be noted that these authors observed an attenuation of the *expression* of morphine tolerance with the antibody to OFQ/N. The antibody was administered once only, on the day of morphine challenge, whereas we administered OFQ/N repeatedly during the development phase, but not on the test day. Our data are, in fact, consistent with the idea that repeated morphine administration repeatedly activates the endogenous OFQ/N system as a homeostatic mechanism, and that a resultant sensitized response of this system upon morphine challenge is responsible for morphine antinociceptive tolerance. Thus, repeated OFQ/N administration during the *development* phase may reduce the activation of endogenous OFQ/N biosynthesis/release, essentially blocking the progressive sensitization of the OFQ/N system observed in the previous investigation (Yuan *et al*., 1999). This would result in an attenuated endogenous OFQ/N response to a subsequent morphine challenge. Alternatively, high concentrations of exogenously applied OFQ/N may cause long-term down-regulation/desensitization of the ORL-1 receptor. Both would potentially be manifested as attenuated antinociceptive tolerance to morphine.

Interestingly, repeated OFQ/N administration in conjunction with saline, as opposed to morphine, did not affect the antinociceptive response to a subsequent morphine challenge. Neither did it affect baseline hot plate latencies. Thus, repeated OFQ/N selectively interfered with the processes involved in the development of morphine tolerance. Given the known action of acute OFQ/N administration to attenuate morphine-induced antinociception (Mogil *et al*., 1996; Morgan *et al*., 1997; Tian *et al*., 1997; Lutfy *et al*., 1999; Wang *et al*., 1999), and the recently reported potentiation of morphine analgesia by an ORL-1 receptor antagonist (Rizzi *et al*., 2000), one would predict that if repeated OFQ/N administration causes desensitization/downregulation of the ORL-1 receptor, such treatment should potentiate the antinociception associated with a subsequent first exposure to morphine. The lack of such an action renders an effect on biosynthesis/release more likely wherein repeated OFQ/N has little effect alone (and therefore the endogenous OFQ/N response to a subsequent initial morphine challenge is unaffected) but prevents the upregulation of OFQ/N biosynthesis in response to repeated morphine.

A more simplistic explanation for our findings is that each OFQ/N administration attenuates the antinociceptive effect of morphine (Mogil *et al*., 1996; Morgan *et al*., 1997; Tian *et al*., 1997; Lutfy *et al*., 1999; Wang *et al*., 1999), thereby preventing induction of events leading to tolerance, events that may not involve the endogenous OFQ/N system itself. However, this is unlikely since there is sufficient evidence to dissociate analgesia itself from analgesic tolerance, i.e., morphine tolerance has been reported in the presence of opioid receptor blockade by naltrexone (Yoburn *et al*., 1990).

It is plausible, however, that exogenous OFQ/N interferes with mechanisms of tolerance independent of its anti-opiate action and independent of an action on endogenous OFQ/N biosynthesis. For instance, learning and memory may contribute significantly to the development of morphine tolerance (Siegel, 1976). Previous studies have shown that analgesic tolerance readily develops after chronic administration of morphine when rats are tested in the same environment in which they received their daily morphine treatment, whereas tolerance is less evident when rats are tested in a novel environment (Siegel, 1976). This phenomenon is referred to as associative tolerance and a Pavlovian conditioning model has been suggested to account for it (Siegel, 1976). Long-term potentiation (LTP) in the hippocampus is implicated as a basis of memory formation, and thus may be important for associating environmental cues with morphine injection. Mediators of LTP include the N-methyl-D-aspartate (NMDA) receptor and calcium/calmodulin-dependent protein kinaseII (CaMKII), both of which are present in high concentrations in the hippocampus (Fan *et al*., 1999). Evidence for their necessity in the development of tolerance is drawn from observations that NMDA receptor antagonists (Marek *et al*., 1991; Trujillo & Akil, 1991; Lutfy *et al*., 1993) and CaMKII inhibitors (Fan *et al*., 1999) attenuate morphine tolerance. Interestingly, OFQ/N has been shown to block LTP in the hippocampus (Yu *et al*., 1997) and intra-hippocampal injection of OFQ/N impairs spatial learning in rats (Sandin *et al*., 1997). Moreover, OFQ/N has been shown to block the effects of morphine in the classical conditioned place preference paradigm (Murphy *et al*., 1999; Ciccocioppo *et al*., 2000) without producing any rewarding or aversive effect of its own (Devine *et al*., 1996). We observed an almost complete blockade of morphine tolerance after repeated OFQ/N administration into the CA3 region of the hippocampus using the same dose that only partially attenuated tolerance after i.c.v. administration. Our data is therefore consistent with the notion that OFQ/N may be acting in the hippocampus to attenuate morphine tolerance, perhaps blocking the associative component of this phenomenon. The lack of complete blockade of morphine tolerance by OFQ/N in these studies presumably reflects the presence of additional tolerance mechanisms (perhaps the non-associative component) that remain unaffected by OFQ/N treatment. Alternatively, as previously demonstrated (Lutfy *et al*., 1999), tolerance develops to the action of OFQ/N and therefore OFQ fails to completely block morphine tolerance.

In conclusion, our results demonstrate that chronic administration of OFQ/N attenuated the development of morphine tolerance in rats after i.c.v. or intra-hippocampal administration without having any significant action on basal nociceptive responses or on acute morphine-induced antinociception.

We are grateful to Dr Shridhar Narayanan for helpful discussions and to Daisuke Arai and Gulpana Mohammad for technical assistance. K. Lutfy was supported by NIDA grant DA00411 and a NARSAD Young Investigator Award. Special thanks to Mr and Mrs Staglin for their support of the NARSAD grant.

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(Received March 13, 2001
Revised July 5, 2001
Accepted July 5, 2001)